The plant homeodomain (PHD) finger is identified in many chromatin-binding proteins, and functions as a ‘reader’ that recognizes specific epigenetic marks on histone tails, bridging transcription factors and their associated complexes to chromatin, and regulating gene expression. PHD finger-containing proteins perform many biological functions and are involved in many human diseases including cancer. PHF14 is predicted to code for a protein with multiple PHD fingers. However, its function is unidentified. The aim of this study is to characterize PHF14 and investigate its biological significance by employing multiple approaches including mouse gene-targeting knockout, and molecular cloning and characterization. Three transcripts of PHF14 in human cell lines were identified by reverse transcriptase polymerase chain reaction. Two isoforms of PHF14 (PHF14α and PHF14β) were cloned in this study. It was found that PHF14 was ubiquitously expressed in mouse tissues and human cell lines. PHF14α, the major isoform of PHF14, was localized in the nucleus and also bound to chromatin during cell division. Interestingly, co-immunoprecipitation results suggested that PHF14α bound to histones via its PHD fingers. Strikingly, gene-targeting knockout of PHF14 in mice resulted in a neonatal lethality due to respiratory failure. Pathological analysis revealed severe disorders of tissue and cell structures in multiple organs, particularly in the lungs. These results indicated that PHF14 might be an epigenetic regulator and play an important role in the development of multiple organs in mouse.

Keywords  plant homeodomain (PHD) finger; PHF14; epigenetic; knockout mice

Introduction

The plant homeodomain (PHD) finger is a common short domain of ~50–80 amino acids (aa), coordinating two zinc ions via the Cys4-His-Cys3 signature in a cross-brace coordination scheme, which is found throughout the eukaryotic genomes from yeast to human [1]. Though the functions of the PHD fingers remain to be fully understood, these proteins are thought to be a diverse group of transcriptional regulators possibly affecting eukaryotic gene expression by influencing chromatin structure. More and more researches have proved that PHD fingers can recognize histone codes in a modification- and context-specific fashion and translate them into biological consequences [2–5], indicating a vital role of PHD finger proteins in regulating cellular epigenetic events. Different PHD finger proteins, such as inhibitor of growth (ING) proteins, PHF2/PHF8 and the autoimmune regulator (AIRE), specifically recognize different ‘histone marks’ and regulate gene transcription via either their intrinsic activities or the activity of their interacting partners [6]. ING proteins bound to H3K4me3 via their PHD fingers, and further stabilized/promoted the activity of histone acetyltransferase or histone deacetylase (HDAC) complexes [7,8]. In response to DNA damage, ING2 PHD domain bound to H3K4me3 and stabilized the mSin3a–HDAC1 complex in the promoters of cyclin D1 gene, and finally resulted in the repression of cyclin D1 mRNA expression [7]. Some PHD finger proteins have enzymatic activities. PHF8, containing both the PHD and Jumonji C (JmJC) domains, functions as an H3K9/H4K20 demethylase. It can be recruited to promoters by its PHD domain based on interaction with H3K4me2/3 and removes the repressive H4K20me1 mark from E2F1-regulated gene promoters to positively regulate gene expression, and control G1/S transition [9].
Naturally occurring point mutations, deletions, or chromosomal translocations in the PHD fingers encoded by many genes predispose individuals to a wide range of pathologies, including cancer, mental retardation, and immunodeficiency, underscoring the significance of PHD proteins as epigenetic readers [6]. Mutations of alpha thalassemia/mental retardation syndrome X-linked protein [10], nuclear receptor-binding SET domain protein 1 [11], cAMP response element-binding protein [12] or PHF6 [13] caused severe syndromes characterized by mental retardation and abnormal growth. ING4 was found to regulate brain tumor angiogenesis [14]. Knockdown of PHF8 homolog in zebrafish caused brain and craniofacial defects [9]. These data suggested that the failure to properly interpret epigenetic markers could result in inappropriate activation or inhibition of downstream pathways, leading to diseases.

In this study, we were particularly interested in a new gene, PHF14 (also called KIAA0783) [15], which is predicted to code for a PHD finger protein. PHF14 is conserved from Caenorhabditis elegans to human (Table 1), indicating its important biological role. Yet, little is known about the function of this new gene. We cloned and characterized PHF14 and further studied its role using our PHF14 knockout (KO) mice as a model system. Our data demonstrated that the ubiquitously expressed PHF14 was a novel chromatin-binding protein interacted with histones. Different PHD domains of PHF14 exhibited a distinct affinity to different histone peptides. Depletion of PHF14 caused neonatal lethality probably due to respiratory failure. Structures of tissues and cells in multiple organs including lung and kidney were found to be severely disordered. These results indicated that PHF14 might be an epigenetic regulator and play an important role in the development of multiple organs in mice.

### Materials and Methods

#### Bioinformatics

The PHD fingers were aligned using ClustalW (version 1.83). The GenBank accession numbers for the PHD finger alignment are BPTF (NP_004450.3), ING2 (NP_001555.1), BHC80 (NP_057705.3), AIRE (NP_000374.1), DPF3B-1 (NP_036206.3), and UHRF1 (NP_001041666.1).

#### Antibodies

The rabbit anti-PHF14 and anti-GST polyclonal antibodies were produced in our own laboratory. The rabbit anti-PHF14 was generated against the GST-fused N-terminal 1–160 aa. Anti-PHF14 antisera were first incubated with GST antigen-immobilized glutathione beads to obtain anti-GST antibodies. The flow-through was purified on GST-PHF14 (N-terminal 1–160 aa)-immobilized glutathione beads to obtain anti-PHF14. All other antibodies used in this study were commercial antibodies: anti-β-actin and anti-α-tubulin (Sigma, St Louis, USA); anti-GAPDH (KangChen Bio-Tech, Shanghai, China); anti-histone H3 (Abcam, Cambridge, UK); anti-LaminB (Santa Cruz Biotechnology, Santa Cruz, USA); anti-myc (Upstate, Waltham, USA); and anti-p38 (Cell Signaling, Beverly, USA).

#### Polymerase chain reaction amplification and DNA sequencing

The entire coding regions of the human PHF14α, β, and γ cDNA were amplified by polymerase chain reaction (PCR) using common forward primer: 5′-GCGAATTTCAGGA TCGCAGCTCAAAGAG-3′; specific reverse primer for PHF14α and PHF14γ: 5′-GCTCTAGATTTCGTTTATGT TTTTCTGGTC-3′; specific reverse primer for PHF14β: 5′-GCTCTAGATGAAGGTATCTGACAAGATTTCC-3′ that were based on the deposited PHF14 sequences (GenBank accession No. NM_014660.3 and Ensemble Transcript ID: ENST00000445996). cDNA from HeLa or A549 cells was used as a template. The PCR products were confirmed by sequencing.

#### RNA extraction and real-time PCR analysis

Total RNA was extracted from mouse tissues using Trizol reagent (Invitrogen, Carlsbad, USA) according to the manufacturer’s protocol. Single-pass cDNA was synthesized using the Prime Script RT reagent Kit (TaKaRa, Dalian, China). Real-time PCR was performed in an ABI 7500 Fast real-time PCR system (Applied Biosystems, Foster, USA) using the SYBR Premix Ex Taq kit (TaKaRa). The following primers were used for the real-time PCR: mouse PHF14α, forward 5′-TGATGAATGCAGGCACAGTCGTCGTTCC-3′ and reverse 5′-CTTGGAGAGAGAGGAGGAGGAGTCACTCCATCC-3′; mouse PHF14β, forward 5′-GCTGACGCTGCTCAGTGG-3′.
and reverse 5'-CTTTTGCTTTCCATCCATGC-3'; and mouse GAPDH, forward 5'-TGTGTCGTCGATGATCTGA-3' and reverse: 5'-CCTGTCCTCACACCTTCTTGTA-3'. Using the quantitative real-time PCR method, we calculated and presented the relative mRNA level of mouse PHF14α and mouse PHF14β. GAPDH was used as an internal control.

### Plasmid constructs

The PHF14α/PHF14β PCR products were cloned into the pRK5-RS vector using the EcoRI and XbaI sites. GFP-PHF14α and GFP-PHF14β were cloned into mammalian expression vectors in two steps. First, green fluorescent protein (GFP) was inserted into the pRK5-RS vector using EcoRI and XbaI sites. Next, XbaI and SalI sites were introduced to the 5'- and 3'-ends of the coding sequence of PHF14α/PHF14β by PCR, and the PCR products were then cloned into pRK5-RS-GFP using the XbaI and SalI sites. All of the plasmid constructs were verified by DNA sequencing.

### Cell fractionation

All cells were maintained in Dulbecco’s modified Eagle’s medium (DMEM; Invitrogen) except that DMEM/F12 (Invitrogen) was used for P19 cells, and the cells were incubated at 37°C in 5% CO₂/95% air. All culture media were supplemented with 10% fetal bovine serum (FBS; Invitrogen).

To obtain cytosolic and nuclear protein fractions, cells were scraped off the plates and lysed in hypotonic buffer [10 mM Tris-HCl, 25 mM KCl, 10 mM NaCl, 1 mM MgCl₂, 0.1 mM EDTA, 1 mM NaF (pH 7.2) and protease inhibitor cocktail]. The lysates were passed through a 25-gauge needle five times and were centrifuged at 400 g for 10 min at 4°C. The supernatant was saved as the cytoplasmic extract. The resulting crude nuclear pellets were suspended in hypotonic buffer and centrifuged at 400 g for 10 min at 4°C. The supernatant was combined with the cytosolic extract. The pellet was dissolved in RIPA buffer [50 mM Tris-HCl pH 7.5, 150 mM NaCl, 1% sodium deoxycholate, 4 mM EDTA, 0.1% sodium dodecyl sulfate (SDS), 1% TritonX-100 and protease inhibitor cocktail], sonicated on ice and saved as the nuclear extract. Chromatin-binding proteins and nuclear matrix proteins were fractionated according to the method described previously [16].

### Preparation of whole-cell extracts and immunoprecipitation

HEK293 cells were lysed with immunoprecipitation (IP) lysis buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.1% Tween-20 and protease inhibitor cocktail) for 20 min at 4°C and were sonicated on ice. Subsequently, the cell lysate was centrifuged for 30 min at 16,000 g. The supernatant was incubated with 10 µl of Protein A-agarose beads (Amersham, Buckinghamshire, UK) and 1 µl of anti-PHF14 sera or non-immune sera (as a control). After rotation for 3 h at 4°C, the beads were washed three times with IP lysis buffer, and the immunoprecipitated proteins were eluted from the beads using 2 × SDS loading buffer and then analyzed by western blot analysis. To avoid chromatin-mediated protein interactions, ethidium bromide (EB) was added in IP buffer during the incubation, or DNase I (TaKaRa) digestion was performed after incubation.

### Western blot analysis

Western blot analysis was performed according to conventional methods after the proteins were transferred to a nitrocellulose membrane (Schleicher & Schuell BioScience, Keene, USA). Immunodetection of the proteins was performed using specific antibodies. Densitometric analysis was performed using Quantity One software (Bio-Rad, Hercules, USA).

### Immunofluorescence

Cells grown on coverslips (Fisher Scientific, Pittsburgh, USA) were washed with phosphate-buffered saline (PBS) and were fixed with methanol at −20°C for 3–5 min or fixed with 4% formaldehyde in PBS at room temperature for 15 min. The cells were then permeabilized with 0.2% Triton X-100 in PBS. After being blocked for 30 min with 1% bovine serum albumin in PBS, the coverslips were incubated with primary antibodies overnight at 4°C. The primary antibodies used were a rabbit anti-PHF14 antibody and a mouse anti-tubulin monoclonal antibody (Sigma). The secondary antibodies used were FITC-conjugated goat anti-rabbit IgG and Cy3-conjugated goat anti-mouse IgG (Jackson ImmunoResearch, West Grove, USA). The cells were then counterstained with 4',6-diamidino-2-phenylindole (DAPI; Sigma) and mounted using PERMAFLUOR aqueous mounting medium (Immunootech, Marseilles, France). Samples were analyzed using laser-scanning confocal microscopy (TCS SP2; Leica, Solms, Germany).

### Immunohistochemical detection

Tissues were processed into paraformaldehyde-fixed, paraffin-embedded specimens. Sections were incubated overnight at 4°C with primary antibodies against PHF14 followed by biotinylated secondary antibodies. Immunohistochemical reactions were visualized using peroxidase-conjugated streptavidin, and 3,3'-diaminobenzidine was used as a chromogen. As a final step, the sections were counterstained with hematoxylin and examined.

### Generation of the PHF14 KO mouse

We constructed a targeting vector for homologous recombination by standard procedures [17]. In the targeting vector (designed to allow a conditional knockout of PHF14), we inserted a loxP site that lies 5’ of the third coding exon of the PHF14 gene, and a neomycin cassette flanked by two FRT (FLP-FRT recombination) sites in the 3’ intron, which was

followed by another loxP site. Embryonic stem cells were electroporated with the PHF14 targeting vector, and cell clones were screened by Southern blot analysis to identify clones that resulted from a correct targeting event. PHF14 targeted ES cell clones were then injected into C57BL/6 blastocysts, generating chimeras that transmitted the targeted allele to progeny. The floxed mice were bred with C57BL/6 Ella-cre mice that express the loxP-specific DNA recombinase. Ella drives expression of cre on embryonic days 1 and 2 and therefore results in cre-mediated deletion of germline cells, generating a heritable constitutive gene knockout. Heterozygous PHF14 (PHF14<sup>+/−</sup>) were selected from the offspring of PHF14 flox/flox × Ella-cre crosses. Homozygous PHF14 KO mice (PHF14<sup>−/−</sup>) were generated by intercrosses of heterozygous PHF14 (PHF14<sup>+/−</sup>) KO mice.

For genotyping, tail DNA was analyzed by PCR using several sets of primers. To demonstrate the presence of the floxed exon3, primer LoxptFa, 5′-CTATTTTCTTGGATTATAGATGCA-3′ and primer LoxptRa 5′-GCCTTCTAAAGTTCCAGCTACTAG-3′ were used, amplifying a 356-bp wild-type band and a 457-bp mutant band (loxP, 101 bp extra). To confirm the presence of the FRT, the primer FRTiFa, 5′-TCTCTAACGGCATTCTAATGAG-3′ and primer FRTiRa, 5′-TTGGAAGGAAAAACACATAGGCA-3′ were used, generating a 226-bp band. Excision of exon3 and the FRT by cre-recombinase was analyzed with primer LoxptFa and primer cre-low, 5′-GCCTGCATTACCGGTGATGC-3′ and primer cre-low, 5′-CAGGGTGTATAGCAATCCC-3′.

**Histological analysis**

Littermates from a PHF14<sup>+/−</sup> × PHF14<sup>+/−</sup> cross were sacrificed a few minutes after natural delivery. The genotypes of the mice were analyzed by PCR. The tissues of the newborn were fixed in 4% phosphate-buffered paraformaldehyde overnight for paraffin-embedded sections. Paraffin-embedded sections were cut at 5 μm. Slides were stained with hematoxylin and eosin for histological examination. Collagen fiber deposition in tissue sections was assessed by Masson’s trichrome staining (BASO, Zhuhai, China) according to the manufacturer’s instruction. Knockout efficiency of heterozygous PHF14 KO mice (PHF14<sup>+/−</sup>) and homozygous PHF14 KO mice (PHF14<sup>−/−</sup>) was confirmed by immunocytochemical staining with anti-PHF14 antibody. For each genotype, at least three mice were analyzed for each assay.

**Results**

**Cloning and domain features of PHF14**

In a proteomic analysis searching for novel phosphotyrosine proteins, we detected KIAA0783, which was later called PHF14. According to the UCSC Genome Browser, the human PHF14 gene is predicted to be located at 7p21.3 and exists as a single copy. We identified three transcripts of PHF14 in HeLa cells by RT-PCR. We named them α, β, and γ forms [Fig. 1(A)]. Protein domains were analyzed using SMART and Pfam. PHF14α has four putative PHD fingers and two coiled-coil regions; PHF14β has a shorter and distinct C-terminal sequence (compared with the α and γ forms), and is predicted to have only the first three PHD fingers; and PHF14γ has a shorter and different N-terminal sequence (compared with the other two forms), contains four putative PHD fingers and one coiled-coil region [Fig. 1(A) and Supplementary Fig. S1]. Comparative sequence analysis of the PHF14 PHD fingers with a selection of known PHD fingers demonstrated complete conservation of the zinc-coordinating residues (Cys4-His-Cys3) [Fig. 1(B)] except that the final cysteine is replaced with a histidine (Cys4-His-Cys2-His) in PHD2. The four PHD fingers of PHF14 cannot be classified into the known subclasses [Fig. 1(B)] because they do not contain the conserved residues that are reported to be involved in ligand recognition [4, 6], suggesting that the PHD fingers of PHF14 might have distinct functions from the known histone-binding subclasses. To elucidate the function of the gene, we cloned the open reading frames of two putative transcripts of human PHF14, PHF14α (the longest form containing all the four putative PHD fingers and two coiled-coil regions) and PHF14β (the one has only three PHD fingers).

**PHF14 is ubiquitously expressed**

To check tissue expression of PHF14, quantitative PCR analysis was performed, and results showed that the homologous mouse PHF14 was widely but variably expressed in different mouse tissues. In comparison to the α form (the homologous transcript of human PHF14α), the β form (the homologous transcript of human PHF14β) was expressed at a much lower mRNA level [Fig. 1(D)]. To ascertain PHF14 expression, we generated specific purified antibodies to the N-terminus of PHF14 [Fig. 1(C) and Fig. 2(A)]. Consistent with the mRNA data, we detected PHF14 protein with a size of 170 kDa in the examined mouse tissues, which corresponded to the α form of PHF14 exogenously expressed in 239T cells [Fig. 1(E)]. High expression levels of PHF14 were detected in the testis, lung and spleen, and low levels were present in the muscle, heart, intestine, and kidney. As shown in Fig. 1(F), PHF14 was also detected in several human tumor cell lines of different tissue origins except HCT-116, a colon carcinoma cell line, which was reported to have a bi-allelic inactivating mutation in its PHF14 gene [18]. Interestingly, in addition to the 170-kDa band, a 155-kDa band (corresponding to the exogenously expressed β isoform of PHF14) was detected in P19 cells, a pluripotent embryonal carcinoma cell line [Fig. 2(C)]. The difference
between theoretical molecular weight (107 kDa for PHF14a or 100 kDa for PHF14β) and the apparent molecular weight likely reflects the post-translational modification of PHF14 since potential N-glycosylation sites and numerous serine/threonine/tyrosine phosphorylation sites were identified by PROSITE. Collectively, PHF14 is ubiquitously but variably expressed in mouse tissues and human tumor cell lines, and PHF14a expresses at higher level than PHF14β.

**PHF14α localizes to the nucleus while PHF14β localizes to the cytoplasm**

We overexpressed PHF14α and β in HCT-116 cells (which have no endogenous PHF14) [Fig. 2(B), left panel] or HeLa cells [Fig. 2(B), right panel], and found that exogenously expressed PHF14α or GFP-PHF14α exclusively localized in the nucleus, while PHF14β or GFP-PHF14β exhibited specific cytoplasmic localization. The P19, 293T, and HeLa cell

Figure 2 PHF14 was associated with chromatin  
(A) The specificity of the PHF14 polyclonal antibody in immunofluorescence analysis. Staining of PHF14 in HeLa cells using the anti-PHF14 polyclonal antibody before (upper) or after (lower) pre-incubation with the antigen. (B) Subcellular localization of overexpressed PHF14. HCT-116 (left panel) or HeLa (right panel) cells were transiently transfected with the indicated constructs. PHF14 was recognized by anti-PHF14 antibody (left panel) or visualized by GFP expression (right panel). Nuclei were visualized with DAPI. Scale bar = 10 μm. (C) Subcellular localization of endogenous PHF14. Left panel: Cell fractionation was performed to determine the endogenous PHF14 localization. Equal amounts of total cell lysate (TCL), the cytoplasmic fraction (Cyto) and the nuclear fraction (Nucl) from each sample were analyzed by western blot analysis. β-Actin and lamin B were used as markers of the cytoplasm and nuclear fractions, respectively. Overexpressed PHF14α and PHF14β in 293T cells served as the control for isoform migration in SDS-PAGE. Right panel: 293T, HeLa, and P19 cells were fixed and stained with anti-PHF14 and anti-tubulin antibodies. Nuclei were visualized with DAPI. Scale bar = 10 μm. (D) Subcellular distribution of PHF14 in HeLa cells during cell cycle progression. Non-synchronized HeLa
lines were employed for determining the cellular localization of endogenous PHF14. A cell fractionation assay revealed that PHF14α appeared exclusively in the nuclear fraction in all three of the cell lines. However, PHF14β was detected exclusively in the cytoplasm of P19 and HeLa cells [Fig. 2(C), left panel]. These data were confirmed by immunofluorescence analysis, in which much weaker cytosolic staining was observed in HeLa cells than in P19 cells [Fig. 2(C), right panel]. The nuclear localization of PHF14α may suggest a DNA-related function because PHD fingers are often present in chromatin-binding proteins [19]. Therefore, we traced the overall molecular behavior of PHF14 during the cell cycle in HeLa cells, which primarily expressed the nuclear-located α form. Confocal microscopy analysis revealed that PHF14 was mainly localized in the nuclei of interphase cells [Fig. 2(D)]. In mitotic cells, the PHF14α signal co-localized with condensed chromatin during metaphase and anaphase [Fig. 2(D)]. A chromatin fractionation assay of HeLa and 293T cells demonstrated that the majority of PHF14 was detected in the chromatin-bound fraction co-fractionated with histone (we probed for H3 as an indicator for histone), while a small fraction co-distributed with nuclear matrix proteins [Fig. 2(E)]. These results suggested that PHF14α might be a novel chromatin-binding protein that is associated with chromosomes throughout mitosis.

**PHF14α is a novel histone-binding protein**

To further understand the function of PHF14α, its potential interaction partners in the nucleus were explored using a proteomics approach using a combination of SILAC, immunoprecipitation, and LC-MS/MS. Over 150 potential PHF14-associated proteins were identified and had functions related to DNA repair, transcriptional regulation, and DNA modification, etc. (data not shown). Interestingly, histone proteins were identified among the most abundant proteins. We further investigated whether PHF14α indeed bound to histones. When the total cell lysate from 293 cells (transfected with GFP-PHF14α or GFP alone) was incubated with purified total calf thymus histones, histone H3, H2A, and H2B were co-immunoprecipitated with GFP-PHF14α, but not with GFP alone [Fig. 3(A), left panel]. Similarly, histones H3, H2A, and H2B could be pulled down together with non-tagged exogenous PHF14α using an anti-PHF14 antibody [Fig. 3(A), right panel]. We then investigated whether histones interacted with endogenous PHF14. A co-immunoprecipitation assay demonstrated that PHF14 clearly bound to histone H3 in 293 cells [Fig. 3(B)]. To find out the importance of distinct PHD domains in histone-binding, deletion mutants of PHF14α were used in co-immunoprecipitation assay. The deletion of either PHD1 or PHD3 domain reduced the amount of histone H3 co-immunoprecipitated with PHF14, and loss of both domains completely abolished the interaction [Fig. 3(C)]. These data clearly suggest that PHF14 is a typical PHD finger protein that binds to histones.

**Deletion of PHF14 is lethal and causes multiple organ abnormalities**

Using homologous recombination experiments with the targeting vector described in Fig. 4(A), mutant mice were generated in which the third coding exon of the PHF14 gene was flanked by loxP sites (i.e. was floxed). Crossing the floxed mutants with mice expressing cre-recombinase in the germline caused the excision of the floxed exon to generate PHF14 KO mice. PCR and immunohistochemistry analysis confirmed the depletion of PHF14 expression in KO mice [Fig. 4(B,C)]. PHF14 homozygous KO mice (PHF14−/− mice) died within a few hours after birth, whereas mice of other genotypes survived at the expected Mendelian ratio [Fig. 5(A)]. Heterozygous KO mice (PHF14+/− mice) appeared healthy and fertile. We performed histological study of several important organs (hematoxylin and eosin-stained sections) in the newborn mice. We could observe dramatic abnormalities in multiple organs including lung and kidney [Fig. 5(B,C)]. In newborn PHF14−/− mice, renal tubulointerstitial fibrosis (assessed by Masson’s trichrome staining) and renal tubule damage were detected. The kidneys of PHF14+/− mice showed similar damages yet at lower levels, while the kidneys of wild-type mice appeared normal [Fig. 5(B,C)]. Histological analysis of lung tissue from the newborns demonstrated slight thickening of the alveolar wall in PHF14+/− mice, but severe hypertrophy of the alveolar wall in PHF14−/− mice [Fig. 5(B)]. These alveoli were poorly-developed and incompletely inflated compared with those of normal mice. This could impair the function of lungs in PHF14−/− mice and cause the neonatal death. Indeed, all PHF14−/− neonates exhibited labored breathing (Supplementary Video S1), which is consistent with a very recent report [20]. These results indicated that PHF14 might play an important role in the development of multiple organs in mice.
In the present study, we characterized PHF14 and demonstrated its cellular and tissue distribution. We proved that PHF14 is a novel histone-binding protein interacting with histones via its PHD fingers. Knockout of PHF14 in mice resulted in disorders of tissue and cell structures in multiple organs and neonatal lethality. Our results indicated that PHF14 might be an epigenetic regulator that played an important role in the development of multiple organs in mice.

PHF14 is conserved both in sequence and structure from C. elegans to human (Table 1), which implies an essential role(s) and biological significance. In this study, three isoforms were identified in human. To explore the function of this gene, we cloned two representative transcripts of PHF14 that have corresponding homologs in mouse [20].

Discussion

In the present study, we characterized PHF14 and demonstrated its cellular and tissue distribution. We proved that PHF14 is a novel histone-binding protein interacting with histones via its PHD fingers. Knockout of PHF14 in mice resulted in disorders of tissue and cell structures in multiple organs and neonatal lethality. Our results indicated that PHF14 might be an epigenetic regulator that played an important role in the development of multiple organs in mice.

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Figure 3 PHF14 interacted with histones (A) PHF14α was immunopurified from 293 cells transiently overexpressing exogenous PHF14α (right panel) or GFP-PHF14α (left panel). The interaction of PHF14 with calf thymus histones are visualized by Coomassie staining. (B) Co-immunoprecipitation of endogenous PHF14 and histone H3. The 293 cell lysate was incubated with non-immune sera (NS) or the anti-PHF14 antibody, and the immunoprecipitated proteins were detected using anti-PHF14 and anti-histone H3 antibodies. EB or DNase I was added to block any DNA-mediated protein interactions. (C) Wild-type (WT) PHF14 and different PHD deletion mutants associated with H3 with different affinities. Exogenously expressed myc-tagged WT PHF14 or different ΔPHD mutants were immunoprecipitated from HCT-116 cells, and the precipitates were analyzed by immunoblotting using anti-H3 and anti-myc antibodies.
We found that PHF14a was the major isoform at the protein level in most of the examined cell lines and adult mouse tissues, implying a general role for PHF14a in different tissues. In contrast to PHF14a, PHF14b protein was almost undetectable in the selected cell lines and in adult mouse tissues except in the embryonal carcinoma P19 cells. PHF14b mRNA, however, could be detected at a low level. This might suggest that the expression of PHF14b is time- and/or tissue-specific. The cytoplasmic localization of PHF14b was somehow unexpected. Using protein subcellular localization prediction tools (e.g. PSORT), we found three putative bipartite NLSs in PHF14b, and four NLSs in PHF14a [Fig. 1(A)]. The different subcellular localization of the two isoforms might suggest that either the fourth NLS (which is missing in PHF14b) is the only properly located NLS (e.g. cell surface-located), or this last NLS is indispensable for the cooperation between the NLSs in nuclear transporting. The cytoplasmic localization of PHF14b implies its distinct role(s) from PHF14a. It is difficult to speculate the function of PHF14b. A few PHD proteins, such as Jade-1 [21,22], Asr1p [23], and MEKK1 [24], were found to localize to the cytosol for an unknown function. Although MEKK1 was shown to function as E3 ubiquitin ligase, its ‘PHD finger’ is suspected to actually be an atypical member of the RING finger family. Whether the PHDs of PHF14b bind to other protein(s) and play a role in the cytosol remains to be clarified. One possibility is that the PHDs of PHF14 have multiple functions because PHD fingers have been found to bind to proteins other than histones [25,26].

Our data support the idea that PHF14 belongs to the PHD finger protein family. All of its four PHD domains are conserved from Drosophila to human, underscoring their importance. The PHD finger can recognize histone markers in a modification- and context-specific fashion and translates them into biological consequences [2,3]. We found that PHF14 interacted with all histones. Due to the lack of an
anti-histone H2 antibody that is successful for western blot analysis, we only investigated the interaction between PHF14 and histone H3 in further detail. A co-immunoprecipitation assay suggested that PHF14 interacted with histone H3 via its PHD1 and PHD3 domains. PHD fingers have been reported to bind to H3K4me3/H3K4me0, the methylation state of H3R2, or the acetylation state of H3K14 on histone H3 to regulate transcription [4, 5, 7–9, 19]. The PHD domains of PHF14 do not belong to any of the known sub-classes, which suggests that the PHF14 domains may preferentially bind to other histone modifications. Histone modifications are known to function in a combinatorial fashion to determine the overall outcome of gene expression. The combinatorial codes can be read by two different domains (e.g. the PHD and bromodomains) of a single code reader or by two readers in a single complex [27–29]. The two histone-binding PHD domains of PHF14 may co-operate in recognizing combinatorial codes, as was recently reported for DPF3B (D4, zinc and double PHD fingers, family 3 protein), in which the first PHD recognizes H3K14ac, and the second PHD recognizes H3K4me0 and H3R2me0 [30].

Figure 5 PHF14 depletion caused abnormalities in multiple organs and was perinatal lethal (A) PHF14 depletion was lethal. The numbers of survived offspring of different genotypes at P0 or 4 week after birth were displayed. (B) Hematoxylin/eosin staining of paraffin sections of PHF14+/+, PHF14+/−, and PHF14−/− mice. Sections of lung, liver, and kidney of mice at P0 were stained. Scale bar = 100 μm. (C) Masson’s trichrome staining of kidneys from PHF14+/+, PHF14+/−, and PHF14−/− mice. Light blue staining indicates the area of collagen deposition. Scale bar = 100 μm.
Once it recognizes specific histone modification, a PHD finger protein will recruit other effectors to regulate gene transcription if it does not have another functional domain of its own [6]. The PHD domains or coiled-coil regions in PHF14 may serve as binding sites for partner effector proteins, such as other transcriptional factors and histone (de)acetylases. Over 150 potential PHF14-associated proteins were identified with functions related to transcriptional regulation, DNA repair, and DNA modification, etc. (data not shown) by MS. In further studies, it would be interesting to study which histone modification(s) PHF14 recognizes in vivo and to investigate whether PHF14 affects DNA/histone modification and how it regulates gene transcription.

To explore the biological function of this novel PHD finger protein, we generated the PHF14-null mice. Knockout of PHF14 is neonatal lethal. Histological analysis revealed that the wall of alveolus was significantly thickened; alveoli were poorly developed and incompletely inflated, which might result in impaired lung function. A very recent study using their own PHF14 KO mice, which also targeted exon3, reported similar findings [20]. The authors further explored the molecular mechanism. They revealed a role of PHF14 in mesenchymal cell proliferation via regulating the expression of PDGFRa. Using ChIP assay they could demonstrate multiple binding sites for PHF14 around the transcription start site of PDGFRa. Yet, no direct binding between PHF14 and the oligonucleotides had been detected. We suspect that PHF14 binds to certain histone methylation at the promoter region of the PDGFRa gene and further recruits other transcriptional factors to suppress its expression. PHD finger protein ING2 was reported to repress cyclin D1 expression using a similar mechanism [7]. Interestingly, we observed abnormality of renal tubule and tubulointerstitial fibrosis in newborn PHF14+/− mice. PDGF receptors are expressed in most of renal cells. And they were reported to be involved in renal fibrosis [31]. This might suggest that PHF14 affected PDGFRa expression in more than one tissue types. Another possibility is that PHF14 regulates multiple genes that share similar histone modification and thus plays a general role in regulating proliferation of different cell types. Our preliminary peptide pull-down assay suggested that PHF14 might bind to several different modifications of H3 peptides by different PHD domains (data not shown). In this way, the number of candidate genes can be regulated by PHF14 will even increase. Indeed, it is unlikely that PDGFRa is the only gene regulated by PHF14. Note the different abnormalities that had been observed in multiple organs, we will further study which genes are regulated by PHF14, and how they contribute to the development of the organs.

In summary, PHF14 is a new PHD finger protein that binds to histones via its PHD domains. Knockout of PHF14 in mice resulted in multiple organ abnormalities and neonatal lethality. Our results indicated that PHF14 might be an epigenetic regulator which had an important role in the development of the mouse.

**Supplementary data**

Supplementary data are available at *ABBS* online.

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